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Award Number: W81XWH-04-1-0387

TITLE: DETERMINING THE EFFECT OF CRYPTOCHROME LOSS AND CIRCADIAN CLOCK DISRUPTION ON TUMORIGENESIS IN MICE

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REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2007	Annual Summary	27 Feb 04 – 26 Feb 07

4. TITLE AND SUBTITLE
DETERMINING THE EFFECT OF CRYPTOCHROME LOSS AND CIRCADIAN CLOCK DISRUPTION ON TUMORIGENESIS IN MICE

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0387

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina
Chapel Hill North Carolina 27599-1350

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Circadian clock disruption may be correlated to increased risk of breast cancer in humans and has been linked to tumor progression and development in mice. We have previously reported that circadian clock disruption by loss of Cryptochrome expression does not cause DNA damage checkpoint or repair defects in mammalian fibroblasts, nor does it cause an increase in tumor predisposition or sensitivity to ionizing radiation in mice. Also of interest is the effect of Cryptochrome loss on gene expression; specifically, we are interested in the role CRYPTOCHROME protein plays in inhibition of circadian gene expression. We find that CRY does not affect the DNA binding of the circadian transcriptional activator CLOCK342-BMAL1, consisting of a 342-amino acid fragment of mammalian CLOCK and full-length BMAL1. In addition, preliminary results indicate that CRY does not affect the DNA binding of CLOCK-BMAL1, consisting of full-length mammalian CLOCK and BMAL1 proteins; however, these results are not yet entirely conclusive and will be investigated further in the future.

15. SUBJECT TERMS
Circadian, clock, Cryptochromes, DNA damage checkpoint, tumorigenesis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

The circadian clock and the cell cycle are two global oscillatory systems in most eukaryotes which are responsible for regulation of many essential pathways and processes. Recent epidemiological and animal model studies indicate that circadian clock disruption can result in increased cancer risk and sensitivity to DNA damaging agents (Hansen, 2001; Filipski et al., 2002; Filipski et al., 2004; Fu et al., 2002). However, our data indicate that circadian clock disruption per se does not predispose to cancer, as mice rendered arrhythmic through loss of both Cryptochrome (CRY) proteins possess intact DNA damage response and are not prone to cancer (Gauger and Sancar, 2005). It is likely that distinct pathways that are disrupted in mutants of certain circadian genes are responsible for the modulation of cancer predisposition. Therefore, the particular effects of circadian clock protein deficiency on cellular gene expression are of interest. Though the current mammalian clock model posits that CRY represses the CLOCK-BMAL1 transcriptional activation complex (Gauger and Sancar, 2005), the mechanism by which this occurs is unknown. Because CRY physically interacts with both CLOCK and BMAL1 (Sancar, 2000; Lee et al., 2001), direct modulation of DNA binding of the CLOCK-BMAL1 complex is a possible mechanism of repression by CRY. Therefore, we examined the effect of CRY on CLOCK-BMAL1 DNA binding using purified proteins in *in vitro* gel mobility shift assays. There are 3 possible ways for CRY to directly affect CLOCK-BMAL1's DNA binding: (1) CRY may cause a conformational change in the heterodimer, possibly by association with the heterodimer, that results in the heterodimer no longer binding to DNA; this mechanism is indicated in the *Drosophila* clock system (Lee et al., 1999); (2) CRY may form a large complex on DNA with CLOCK and BMAL1 that is no longer transcriptionally active; this mechanism is indicated in the zebrafish clock system (Ishikawa et al., 2002); (3) CRY may replace one of the components of the heterodimer to form a transcriptionally inactive complex on DNA. Figure 1 shows a cartoon schematic of these mechanisms.

The specific aims of this proposal are as follows:

1. **We will determine the effect of circadian clock disruption on uninduced tumor development in cryptochrome-deficient mice.**

2. **We will determine the susceptibility of mice with disrupted circadian rhythms to ionizing radiation.**

3. **We will use cell-based assays to investigate the cell cycle phenotype of cryptochrome-deficient cells under normal and induced conditions.**

4. **(additional) We will investigate the effect of cryptochrome loss on gene expression by analyzing the effect of CRY on DNA binding of the circadian transcriptional activator CLOCK-BMAL1.** (Note: This aim was not explicit in the original proposal but was determined to be a necessary part of this work over the course of other experiments.)

Specific Aim 1: We will determine the effect of circadian clock disruption on uninduced tumor development in cryptochrome-deficient mice.

Completed and reported as of 2006 annual summary

Specific Aim 2: We will determine the susceptibility of mice with disrupted circadian rhythms to ionizing radiation.

Completed and reported as of 2006 annual summary

Specific Aim 3: We will use cell-based assays to investigate the cell cycle phenotype of cryptochrome-deficient cells under normal and induced conditions.

Completed and reported as of 2006 annual summary

Specific Aim 4: (additional) We will investigate the effect of cryptochrome loss on gene expression by analyzing the effect of CRY on DNA binding of the circadian transcriptional activator CLOCK-BMAL1.

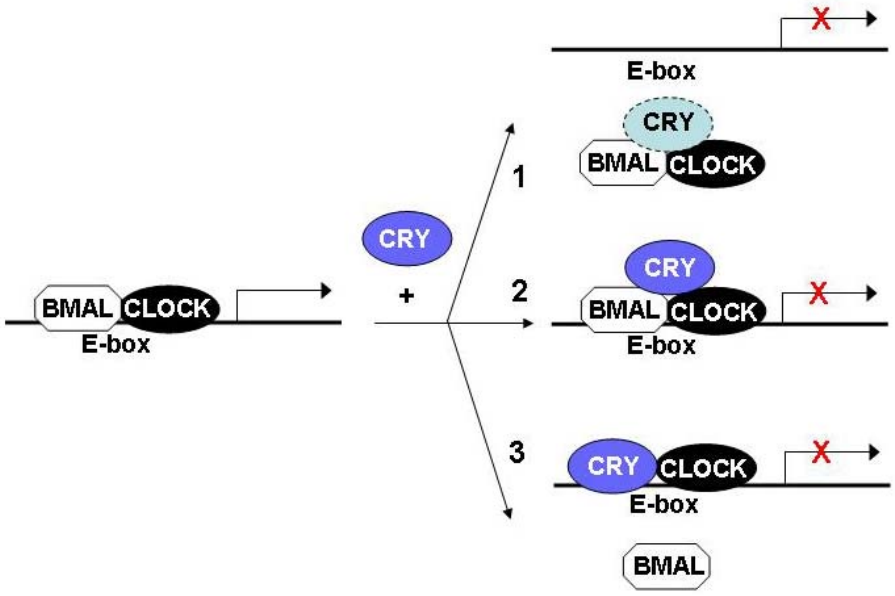

Figure 1**.** Possible Mechanisms for CRY's Negative Regulation of CLOCK-BMAL1 by Directly Affecting DNA Binding. CRY may directly affect CLOCK-BMAL1 by **(1)** changing the conformation of CLOCK-BMAL1, possibly by forming a trimeric complex, such that the complex no longer binds to DNA; **(2)** forming a trimeric complex on DNA which no longer is transcriptionally active; or **(3)** replacing one of the components of the CLOCK-BMAL1 heterodimer to form a transcriptionally inactive complex on DNA.

The mammalian circadian clock proteins CLOCK, BMAL1, and CRY1 were affinity-purified from baculovirus-infected insect cells (CLOCK, BMAL1) and transiently-transfected 293T fibroblasts (CRY1). In addition, a 342-amino acid fragment of CLOCK was expressed and purified from inclusion bodies in *E. coli*; this protein has been used successfully in DNA binding assays with full-length BMAL1 and performs similarly to full-length CLOCK (Rutter et al., 2001; previous unpublished results from our laboratory). Figure 2A shows a silver-stained SDS-PAGE of the purified proteins.

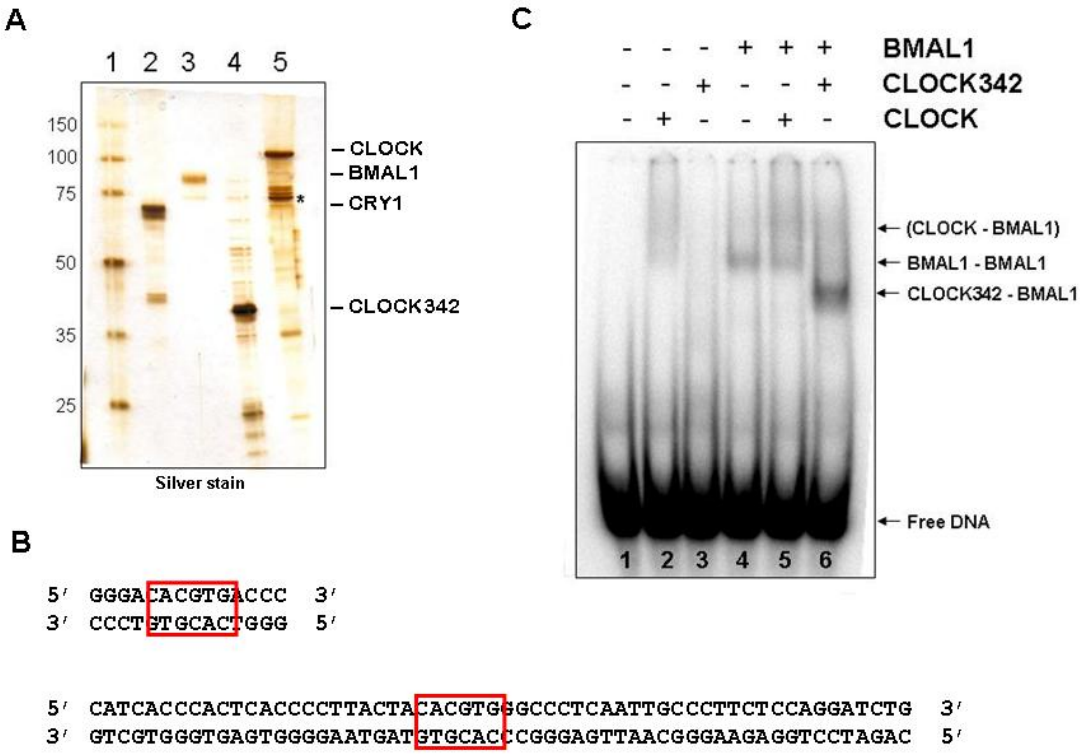

Figure 2. Purified circadian clock proteins and DNA binding to E-box DNA substrates. (A) SDS-PAGE of clock proteins. Lane 1: molecular weight marker. Lane 2: CRY1 protein purified from transiently transfected 293T fibroblasts. Lane 3: BMAL1 protein purified from baculovirus-infected insect cells. Lane 4: CLOCK342 protein purified from *E. coli*. Lane 5: CLOCK protein purified from baculovirus-infected insect cells. * denotes a contaminant protein, likely heat-shock protein. (B) Double-stranded (ds) 14mer (above) and 58mer (below) E-box DNA substrates used in gel mobility shift assays. E-box elements are outlined in red. (C) Gel mobility shift assay using ds 58mer DNA substrate and purified proteins. Arrows indicate protein-DNA complexes.

The purified clock proteins were used in gel mobility shift assays with either a ds 14mer or a ds 58mer DNA substrate containing a single E-box element, CACGTG (Figure 2B). Figure 2C shows formation of the CLOCK342-BMAL1 heterodimer on DNA (lane 6). Additionally, lane 4 of Figure 2C shows the formation of what is thought to be a BMAL1-BMAL1 homodimer on DNA (Rutter et al., 2001). We were unable to detect discrete formation of a heterodimer of full-length CLOCK and BMAL1 on DNA (lane 5).

The effect of CRY on the DNA binding of CLOCK-BMAL1 and CLOCK342-BMAL1 was examined. As seen in Figure 3, CRY1 is also a DNA-binding protein, although this binding is thought to be nonspecific (Ozgur and Sancar, 2003) while the DNA binding of CLOCK-BMAL1 and CLOCK342-BMAL1 is specific to E-box DNA elements. Although we could not detect a discrete heterodimer of full-length CLOCK-BMAL1 on DNA, it appeared that CRY1 did not change the slight smear pattern thought to be CLOCK-BMAL1 on DNA (Figure 3).

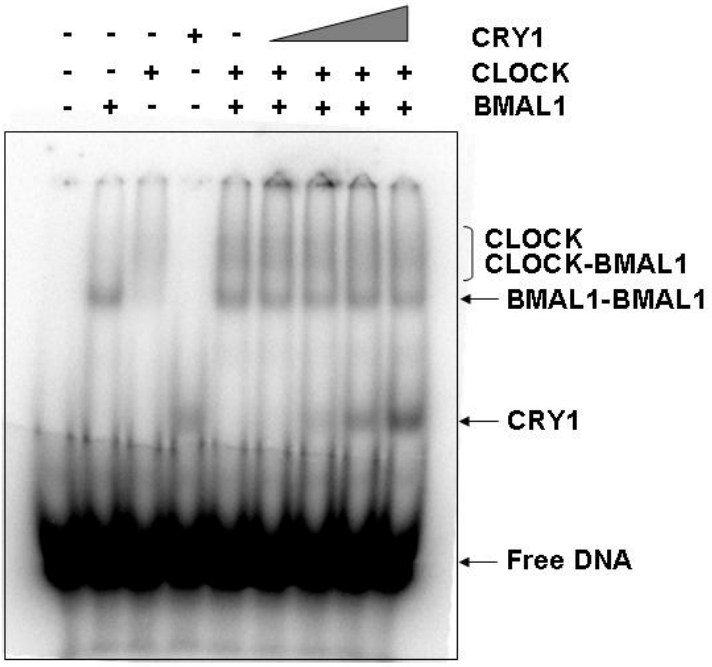

Figure 3. Effect of CRY1 on DNA binding of CLOCK-BMAL1 heterodimer to DNA. Gel mobility shift assay was performed using ds 58mer DNA substrate. Arrows and brackets indicate protein-DNA complexes.

Because we were able to better detect the formation of a heterodimer of CLOCK342-BMAL1 on DNA, we also examined the effect of CRY on the DNA binding of this complex. Because CRY1 is itself a DNA binding protein, this experiment was performed under conditions of high-affinity CRY DNA binding and low-affinity CRY DNA binding. Again, we do not see any effect of CRY on the DNA binding of CLOCK342-BMAL1 (Figure 4).

KEY RESEARCH ACCOMPLISHMENTS

- CRY1 is a DNA binding protein that likely binds to DNA in a non-specific manner (Specific Aim 4).
- CRY1 does not observably affect the DNA binding of the CLOCK-BMAL1 heterodimer (Specific Aim 4).
- CRY1 does not observably affect the DNA binding of the CLOCK342-BMAL1 heterodimer (Specific Aim 4).

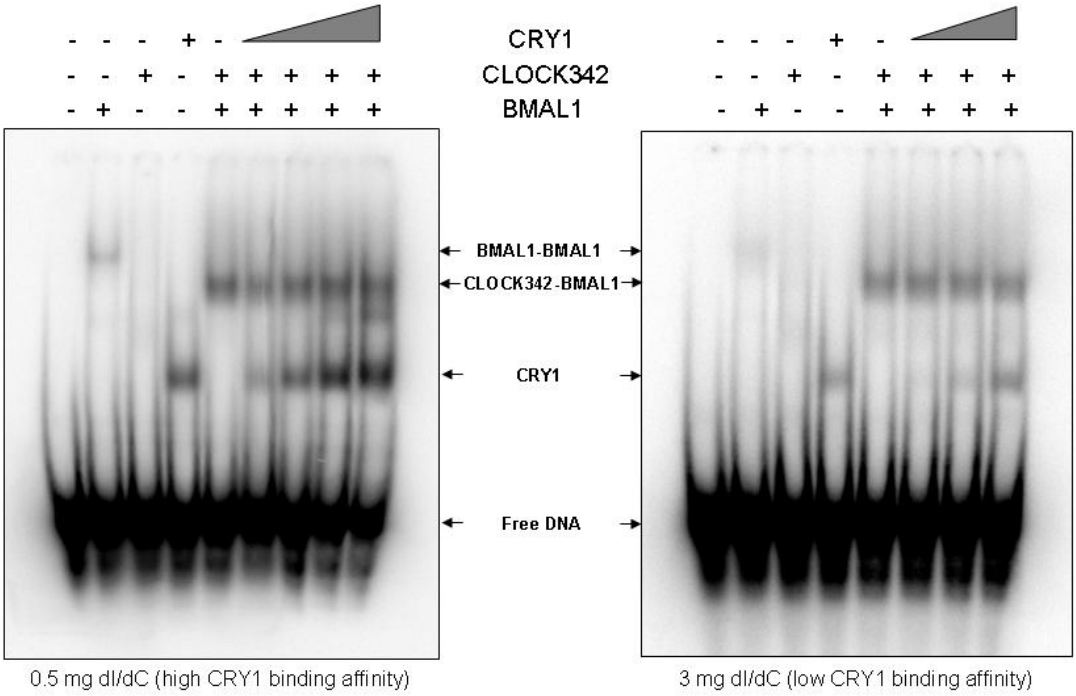

Figure 4. Effect of CRY1 on Binding of CLOCK342-BMAL1 to E-box DNA. EMSAs were carried out as described using E58 ds radiolabeled DNA under conditions of either high CRY1 DBA-binding affinity (left panel) or low CRY1 DNA-binding affinity (right panel). Arrows indicate the binding of BMAL1-BMAL1 homodimer, CLOCK342-BMAL1 heterodimer, or CRY1 to DNA.

REPORTABLE OUTCOMES

- PRESENTATION
  Gauger MA. Cryptochrome, circadian clock, cell cycle, and cancer. Dissertation defense, University of North Carolina at Chapel Hill, Department of Biochemistry and Biophysics, March 26, 2007.

- PUBLICATION
  Gauger MA and Sancar A. Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Dissertation.* University of North Carolina at Chapel Hill, Department of Biochemistry and Biophysics.

CONCLUSIONS

Our current progress on the work outlined in our proposal leads us to conclude that, by detection using the gel mobility shift DNA binding assay used in the above-described experiments, CRY does not directly affect the DNA binding of the CLOCK-BMAL1 heterodimer nor the CLOCK342-BMAL1 heterodimer. However, these experiments must be extended because there is evidence that CLOCK342 is a physiologically irrelevant protein as it is missing a residue found to be necessary for repression of CLOCK-BMAL1 by CRY (Sato et al., 2006).

Therefore, the somewhat inconclusive experiments using full-length CLOCK must be followed up in order to properly address this question.

REFERENCES

1. Filipski, E., King, V.M., Li, X., Iacobelli, S., Xian, L.J., and Levi, F. (2002) Host circadian clock as a control point in tumor progression in mice. *J Natl Cancer Inst*, **94 (9)**, 690-697.

2. Filipski, E., Delaunay, F., King, V.M., Wu, M.W., Claustrat, B., Grechez-Cassiau, A., Guettier, C., Hastings, M.H., and Levi, F. (2004) Effects of chronic jet lag on tumor progression in mice. *Cancer Res*, **64**, 7879-7885.

3. Fu, L., Pelicano, H., Liu, J., Huang, P, and Lee, C.C. (2002) The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo. *Cell*, **111**, 41-50.

4. Gauger, M.A. and Sancar, A. (2005) Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Res*, **65 (15)**, 6828-6834.

5. Hansen, J. (2001) Increased breast cancer risk among women who work predominantly at night. *Epidemiology*, **12 (1)**, 74-77.

6. Ishikawa, T., Hirayama, J., Kobayashi, Y., and Todo, T. (2002) Zebrafish CRY represses transcription mediated by CLOCK-BMAL heterodimer without inhibiting its binding to DNA. *Genes to Cells*, **7**, 1073-1086.

7. Lee, C., Bae, K., and Edery, I. (1999) PER and TIM inhibit the DNA binding activity of a Drosophila CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. *Mol Cell Biol*, **19**, 5316-5325.

8. Lee, C., Etchegaray, J.P., Cagampang, F.R., Loudon, A.S., and Reppert, S.M. (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell*, **107**, 855-867.

9. Ozgur, S. and Sancar, A. (2003) Purification and properties of human blue-light photoreceptor Cryptochrome 2. *Biochemistry*, **42**, 2926-2932.

10. Rutter, J., Reick, M., Wu, L.C., and McKnight, S.L. (2001) Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science*, **293**, 510-514.

11. Sancar, A. (2000) Cryptochrome: the second photoactive pigment in the eye and its role in circadian photoreception. *Annu Rev Biochem*, **69**, 31-67.

12. Sato, T.K., Yamada, R.G., Ukai, H., Baggs, J.E., Miraglia, L.J., Kobayashi, T.J., Welsh, D.K., Kay, S.A., Ueda, H.R., and Hogenesch, J.B. (2006) Feedback repression is required for mammalian circadian clock function. *Nat Genet*, **38**, 312-319.